

High Yields of 2,3-Butanediol and Mannitol in *Lactococcus lactis* through Engineering of NAD⁺ Cofactor Recycling^{▽†}

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Received 24 May 2011/Accepted 2 August 2011

Manipulation of NADH-dependent steps, and particularly disruption of the *las*-located lactate dehydrogenase (*ldh*) gene in *Lactococcus lactis*, is common to engineering strategies envisaging the accumulation of reduced end products other than lactate. Reverse transcription-PCR experiments revealed that three out of the four genes assigned to lactate dehydrogenase in the genome of *L. lactis*, i.e., the *ldh*, *ldhB*, and *ldhX* genes, were expressed in the parental strain MG1363. Given that genetic redundancy is often a major cause of metabolic instability in engineered strains, we set out to develop a genetically stable lactococcal host tuned for the production of reduced compounds. Therefore, the *ldhB* and *ldhX* genes were sequentially deleted in *L. lactis* FI10089, a strain with a deletion of the *ldh* gene. The single, double, and triple mutants, FI10089, FI10089 Δ *ldhB*, and FI10089 Δ *ldhB* Δ *ldhX*, showed similar growth profiles and displayed mixed-acid fermentation, ethanol being the main reduced end product. Hence, the alcohol dehydrogenase-encoding gene, the *adhE* gene, was inactivated in FI10089, but the resulting strain reverted to homolactic fermentation due to induction of the *ldhB* gene. The three lactate dehydrogenase-deficient mutants were selected as a background for the production of mannitol and 2,3-butanediol. Pathways for the biosynthesis of these compounds were overexpressed under the control of a nisin promoter, and the constructs were analyzed with respect to growth parameters and product yields under anaerobiosis. Glucose was efficiently channeled to mannitol (maximal yield, 42%) or to 2,3-butanediol (maximal yield, 67%). The theoretical yield for 2,3-butanediol was achieved. We show that FI10089 Δ *ldhB* is a valuable basis for engineering strategies aiming at the production of reduced compounds.

Lactococcus lactis, a fermentative bacterium used worldwide in the manufacture of dairy products, is among the best characterized species of lactic acid bacteria (LAB). The wealth of knowledge generated in the fields of lactococcal genetics and physiology, combined with a “generally recognized as safe” (GRAS) status, a relatively simple metabolism, and a small genome, has rendered *L. lactis* an attractive model with which to implement metabolic engineering strategies (12, 21, 53).

L. lactis is a homofermentative bacterium, which converts approximately 95% of the sugar substrate to lactic acid. In the last 15 years, numerous attempts have been made to reroute carbon flux from lactate to the production of other organic compounds via metabolic engineering. Manipulation of NADH-dependent steps is common to many of the strategies envisaging such a goal. In particular, disruption of the *las* (lactic acid synthesis) operon-encoded lactate dehydrogenase (LDH), the major player in the regeneration of NAD⁺, is frequent (19, 25, 45, 60). The resultant constraint at the level of NAD⁺ regeneration can be exploited to increase the production of reduced compounds under anaerobic conditions. This approach has been successfully applied in the

production of value-added compounds, such as alanine (25) or mannitol (19, 61).

Generally, *L. lactis* strains in which the *las*-encoded *ldh* gene has been inactivated produce from glucose a mixture of end products, including lactate. The recurrent detection of lactate has been recently attributed to the activity of the *ldhB* gene product (5, 20). Interestingly, inserting insertion elements (IS) in a site-specific and oriented way in the *ldhB* promoter region results in high expression of the gene, and ultimately, this random event could restore a fully homolactic fermentation profile (20). In addition to the *ldhB* gene, two other genes, the *ldhX* and *hicD* (llmg_0475 in MG1363) genes, encoding proteins with at least 30% amino acid sequence identity to LDH, are present in the genome sequences of *L. lactis* (4, 39, 51, 58).

Given that genetic redundancy is often a major cause of metabolic instability in engineered strains which limits their application in industrial fermentations, we set out to develop a genetically stable lactococcal host tuned for the production of biotechnological relevant reduced compounds. To this end, we constructed a series of mutants with deletions in genes encoding NAD⁺ regenerating steps downstream of the pyruvate node (Fig. 1). Our team was the first to report the detection of mannitol as a fermentation product of *L. lactis* LDH-deficient strains (41, 42). However, this polyol was fully catabolized once the sugar substrate was depleted. Thus, as part of our strategy to enhance mannitol production, a first-generation Δ *ldh* Δ *mtlF* strain (FI10089) was constructed, in which LDH and the spe-

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[▽] Published ahead of print on 12 August 2011.

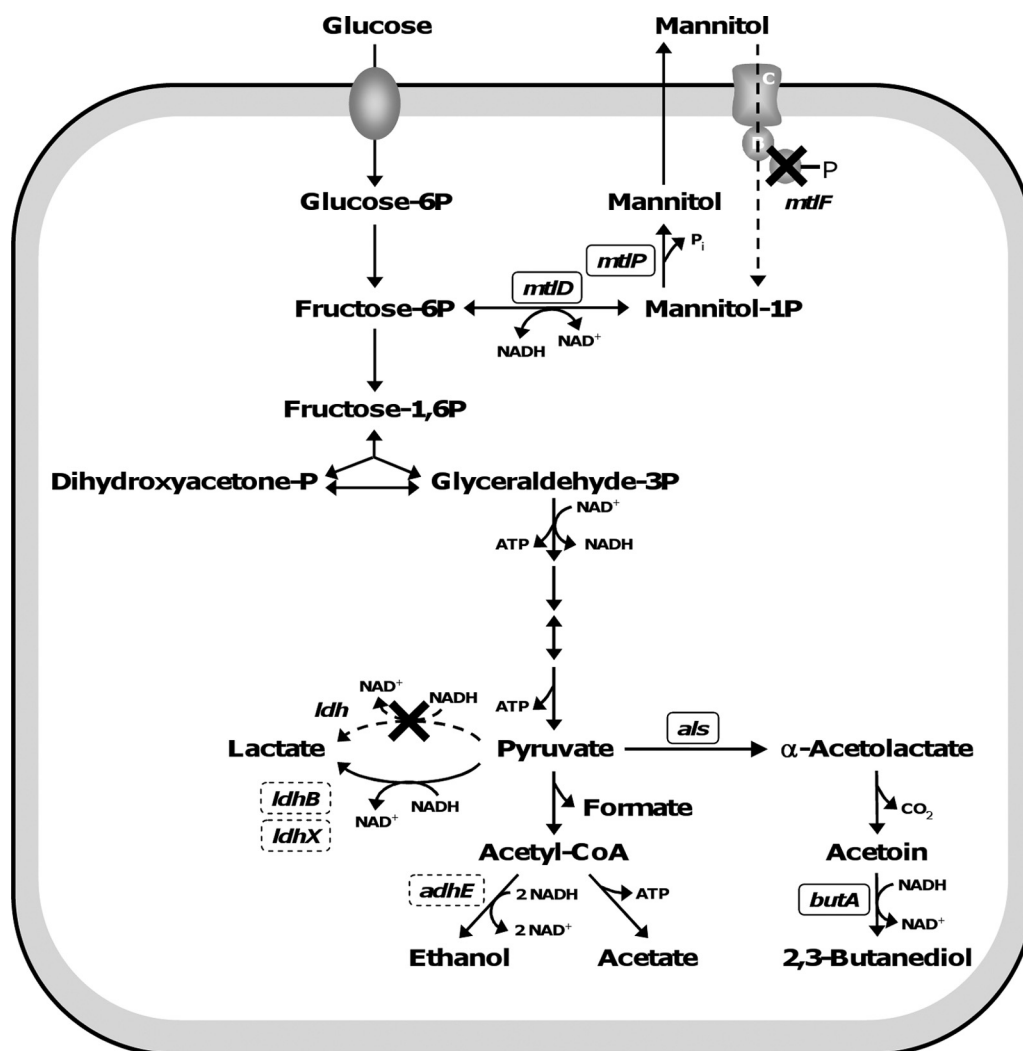


FIG. 1. Schematic representation of glucose metabolism in *L. lactis* FI10089, an *ldh*-deficient strain with a deletion on the mannitol transport system (highlighted with dashed arrows and crosses). Genes deleted or overexpressed in FI10089 are boxed in dashed or unbroken lines, respectively. Gene names and corresponding gene products are as follows: *mtlD*, mannitol 1-phosphate dehydrogenase; *mtlP*, mannitol 1-phosphate phosphatase; *ldhB*, lactate dehydrogenase B; *ldhX*, lactate dehydrogenase X; *adhE*, alcohol dehydrogenase; *als*, α -acetolactate synthase; *butA*, acetoin reductase. Acetyl-CoA, acetyl coenzyme A.

cific enzyme IIA of the mannitol-phosphoenolpyruvate phosphotransferase system are inactivated (19). Here, we used this strain as background for further manipulation aimed at efficient production of polyols. In order to channel the reducing power toward the desired redox reaction, genes encoding known or putative dehydrogenase (*ldhB*, *ldhX*, *llmg_0475*, and *adhE*) genes were chosen for deletion based on the following arguments: (i) ethanol was the major reduced product during the fermentation of glucose by FI10089, accounting for about 40% of the consumed glucose (19); (ii) high-level expression of *ldhB* in LDH-deficient strains can be activated by insertion of IS in its promoter region (20); and (iii) high sequence similarity exists between LDH and the proteins encoded by the *ldhX* and *llmg_0475* genes.

To capitalize on the data obtained, three mutants with inactivation of different lactate dehydrogenase-encoding genes were selected as the most promising hosts. To test their usefulness for the production of reduced, value-added com-

pounds, pathways for mannitol and 2,3-butanediol biosynthesis were overexpressed in each of the three lactate dehydrogenase-deficient strains. The economical value of mannitol and 2,3-butanediol has justified significant research effort to implement their biotechnological production (10, 27, 49, 52). Mannitol is a low-calorie sugar with health-promoting properties (49, 52), and 2,3-butanediol is an important chemical feedstock with a wide range of applications (10, 27). In addition to the biotechnological relevance of mannitol and 2,3-butanediol, these polyols provide interesting case studies in which the major sink for reducing equivalents is located upstream and downstream of the pyruvate node, respectively. Our strategy led to product yields close to the maximal theoretical values.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For a list of strains and plasmids used in this work, see Table S1 in the supplemental material. For molecular biology

procedures, *L. lactis* strains were cultivated as batch cultures (flasks) without aeration in M17 medium (Difco) supplemented with 0.5% glucose (wt/vol) at 30°C or 38°C (gene integration/excision). Reverse transcription-PCR (RT-PCR) studies were performed with cells grown in rubber-stoppered bottles (80 ml) in chemically defined medium (CDM) (46) without pH control (initial pH 6.5). For physiological studies, MG1363 (22), FI10089 (19), and the derivatives FI10089 Δ ldhB, FI10089 Δ ldhB Δ ldhX, and FI10089 Δ adhE were grown in CDM under anaerobic conditions at 30°C in a 2-liter fermentor B. Braun Biostat MD (B. Braun Biotech International, Melsungen, Germany). Glucose was added to a final concentration of 1% (wt/vol), and pH was kept at 6.5 by the automatic addition of 10 M NaOH. Anaerobic conditions were attained by flushing sterile argon through the medium for 1 h preceding inoculation. Strains engineered for the production of mannitol and 2,3-butanediol were grown in rubber-stoppered bottles (100 ml) in CDM without a pH control (initial pH 6.5). Plasmid selection was achieved by addition of 5 mg \cdot liter⁻¹ erythromycin and/or 5 mg \cdot liter⁻¹ chloramphenicol to the growth medium. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) and calibrating against cell dry mass measurements. For controlled overproduction of mannitol 1-phosphate dehydrogenase (MTLD) and mannitol 1-phosphate phosphatase (MTLP) or α -acetolactate synthase (ALS) and acetoin reductase (AR), nisin (1 μ g \cdot liter⁻¹) was added at an OD₆₀₀ of 0.5. Specific growth rates (μ) were calculated through linear regression of the plots of ln(OD₆₀₀) versus time during the exponential growth phase.

Molecular techniques. General molecular techniques were performed essentially as described elsewhere (50). Chromosomal DNA was isolated by the method of Johansen and Kibbenich (28). Plasmid DNA isolation was carried out using a QIAprep Spin Miniprep kit (Qiagen, United Kingdom) for small-scale purifications with the following modifications: the bacterial pellet was resuspended in Birnboim A solution (3), containing 5 mg \cdot ml⁻¹ lysozyme and 30 μ g \cdot ml⁻¹ RNase I, and incubated at 55°C for 10 to 15 min before the addition of buffer P2 (Qiagen, United Kingdom). *L. lactis* was transformed with plasmid DNA by electroporation as described by Holo and Nes (24). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Ipswich, MA) and *Pwo* polymerase and *Taq* polymerase were from Roche Applied Science (Mannheim, Germany), and they were used according to the suppliers' instructions. PCRs were performed with a MyCycler thermal cycler (Bio-Rad, Hercules, CA). Primers (listed in Tables S2 and S3 in the supplemental material) were purchased from Sigma-Genosys (Cambridgeshire, United Kingdom) or Thermo Electron Corporation (Ulm, Germany).

Construction of strains and plasmids. (i) **Gene deletions.** The *ldhB* (llmg_0392), *ldhX* (llmg_1429), and *adhE* (llmg_2432) genes were deleted in *L. lactis* strains by using a two-step homologous recombination method (32). This method does not leave antibiotic resistance markers in the chromosome, and multiple deletions in one strain can be accomplished. Chromosomal DNA of *L. lactis* MG1363 was used as a template in PCR amplifications. For deletion of the *ldhB* gene, the upstream and downstream flanking regions of the gene were amplified using the primer pairs ldhBfo/ldhBri and ldhBfi/ldhBro (see Table S2 in the supplemental material). These oligonucleotides were designed to generate PCR products containing complementary end sequences. Both flanking regions were then spliced by overlap extension in a second PCR as described by Ho et al. (23) and cloned as a BamHI/BglII restriction fragment in pORI280 (32), resulting in plasmid pFI2493. The plasmid was obtained and maintained in LL108 (33). The same strategy was pursued for deletion of the *adhE* (primer pairs adhEfo/adhEri and adhEfi/adhEBro) and *ldhX* (primer pairs ldhX-KO1/ldhX-KO2 and ldhX-KO3/ldhX-KO4b) genes, originating the plasmids pFI2494 and pORI Δ ldhX, respectively. The pFI2493 or pFI2494 plasmids were cotransformed with pVE6007 (38) into FI10089, and the resulting strains were taken through the temperature shift protocol for single and double crossovers, yielding FI10089 Δ ldhB and FI10089 Δ adhE, respectively. Strain FI10089 Δ ldhB Δ ldhX was obtained in a similar manner after cotransformation of pORI Δ ldhX and pVE6007 into FI10089 Δ ldhB, as was FI10089 Δ adhE Δ ldhB after cotransformation of pFI2493 and pVE6007 into FI10089 Δ adhE.

Gene deletions were confirmed by PCR and by Southern blotting. Probe labeling, hybridization, and detection were performed using an ECL direct nucleic acid labeling system, according to the specifications of the manufacturer (Amersham Biosciences, United Kingdom).

(ii) **Overexpression of genes.** The gene encoding MTLP from *Eimeria tenella* (GenBank accession number AF032462.1) and the genes encoding MTLD (*mtlD*, llmg_0025), ALS (*als*, llmg_1309), and AR (*butA*, llmg_1641) from *L. lactis* MG1363 were overexpressed in FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX as follows. The MTLP gene was amplified by PCR from the *Escherichia coli* expression vector pQE-60 (Qiagen, United Kingdom) containing the MTLP-encoding gene from *E. tenella* (35), using the primers Pase-Fw1-

wRBS and Pase-NR (see Table S2 in the supplemental material). Besides the XbaI restriction site, the Pase-Fw1-wRBS primer introduced a consensus lactococcal ribosome binding site (5'-TAAAGGAGG-3') and a 7-base spacer (5'-ATAATA-3') (11) preceding the ATG initiation codon of the *mtlP* gene. The XbaI/HindIII-digested PCR product was cloned into the nisin-inducible expression vector pNZ8048 (31), yielding construct pNZ-*mtlP*. The *mtlD* gene was amplified by PCR from *L. lactis* MG1363 using the primer pair mtlD-fw/mtlD-rev (see Table S2 in the supplemental material). The BspHI/XbaI PCR product was cloned upstream of the *mtlP* gene in NcoI/XbaI-digested pNZ-*mtlP*, resulting in pNZ-*mtlDmtlP*. The *als* and *butA* genes were amplified by PCR from *L. lactis* MG1363, using the primer pairs als1for/als2rev and butA3for/butA2rev, respectively. ButA3for was designed to amplify the native ribosome binding site region upstream of the *butA* start codon. The BsaI/XbaI PCR product containing *als* sequence was cloned in NcoI/XbaI-digested pNZ8048, yielding the construct pNZ-*als*. The XbaI/HindIII PCR product containing the *butA* gene, and the respective ribosome binding site sequence was cloned downstream of the *als* gene in pNZ-*als* digested with the same restriction enzymes, resulting in pNZ-*alsbutA*. The plasmids were obtained and maintained in NZ9000 (31).

The gene sequences of the different constructs were verified by sequencing. pNZ-*mtlDmtlP* or pNZ-*alsbutA* were transformed into FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX, originating FI10089Mtl⁺, FI10089 Δ ldhBMtl⁺, and FI10089 Δ ldhB Δ ldhXMtl⁺ or FI10089BD⁺, FI10089 Δ ldhBBD⁺, and FI10089 Δ ldhB Δ ldhXBD⁺, respectively. Control strains were also constructed by transformation of FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX with empty expression vector pNZ8048, yielding FI10089pNZ, FI10089 Δ ldhBpNZ, and FI10089 Δ ldhB Δ ldhXpNZ. Plasmid pNZ9530 (30), containing *nisR* and *nisK* genes, was cotransformed with pNZ8048 and its derivatives to allow nisin-induced expression of the *mtlD* and *mtlP* or *als* and *butA* genes in the different strains. The *nisRK* genes, coding for the histidine protein kinase NisK and the response regulator NisR, are the only *nis* genes required for *nisA* promoter activation on pNZ-*mtlDmtlP* and pNZ-*alsbutA* plasmids (30).

RT-PCR experiments. *L. lactis* MG1363, FI10089, and derivatives were grown as described above. Total RNA was isolated from cells in the mid-exponential growth phase (OD₆₀₀ = 1) using an SV total RNA isolation system (Promega, Woods Hollow Road, MA), with the following modifications: incubation with lysozyme (5 mg \cdot ml⁻¹, 20 min at 37°C) preceded the first step of the kit protocol, and an additional incubation step with the kit DNase I (1.5 h, 24°C) was required to remove chromosomal DNA. Total RNA (1 μ g), deoxynucleoside triphosphates (dNTPs; final concentration of 0.5 mM) and random oligonucleotides (12 μ g \cdot ml⁻¹) (Invitrogen, Carlsbad, CA) were heated to 65°C for 5 min and chilled on ice. Dithiothreitol (final concentration of 5 mM), first-strand RT buffer, and Superscript III (1:20 [vol/vol]) (Invitrogen, Carlsbad, CA) were added, and samples were incubated for 5 min at 25°C, 60 min at 50°C, and 15 min at 70°C for enzyme inactivation. A parallel sample was treated in the same way, except for the addition of enzyme. cDNA was subsequently used 1:30 (vol/vol) in standard PCRs. To test for contamination of RNA with DNA, the RNA samples without reverse transcriptase were used as negative controls for all conditions tested. Chromosomal *L. lactis* MG1363 DNA was used as positive control for the PCR. The primer pairs (see Table S3 in the supplemental material) were designed to amplify internal *ldh* (*ldh*_RT1/*ldh*_RT2), *ldhB* (*ldhB*_RT1/*ldhB*_RT2), *ldhX* (*ldhX*_RT1/*ldhX*_RT2), llmg_0475 (llmg0475_RT1/llmg0475_RT2), and *tufA* (*tuf*_RT1/*tuf*_RT2) fragments. The *L. lactis* *tufA* (llmg_2050) gene, a housekeeping gene coding for the elongation factor Tu required for continued translation of mRNA, was used as a control. Reverse transcription-PCR was performed three times with RNA isolated from independent cultures.

Quantification of extracellular metabolites. Samples (2 ml) of cultures growing in CDM containing glucose were collected at different points during growth and centrifuged (16,100 \times g, 5 min, 4°C), and the supernatant solutions were stored at -20°C until analysis by high-performance liquid chromatography (HPLC) or ¹H nuclear magnetic resonance (NMR) (43). Fermentation substrate and products were quantified by HPLC as described by Gaspar et al. (19). In supernatants derived from cultures of strains overexpressing the 2,3-butanediol biosynthetic pathway, acetoin quantification was alternatively performed by ¹H-NMR using a Bruker AMX300 spectrometer and a 5-mm inverse detection probe head. The values reported (see Tables 2 and 4) are averages from at least two independent growths.

Enzyme activity measurements. For determination of dehydrogenase-specific activities in MG1363, FI10089, FI10089 Δ ldhB, FI10089 Δ ldhB Δ ldhX, and FI10089 Δ adhE, cells were grown in CDM supplemented with glucose in a 2-liter fermentor as described above, harvested in the mid-logarithmic growth phase (OD₆₀₀ = 2.0), washed twice with 5 mM potassium phosphate (KP_i) buffer, pH 7.2, and resuspended to a protein concentration ranging from 10 to 15 mg \cdot ml⁻¹ in 50 mM KP_i (pH 7.2) or 125 mM triethanolamine (pH 7.2) with 5 mM

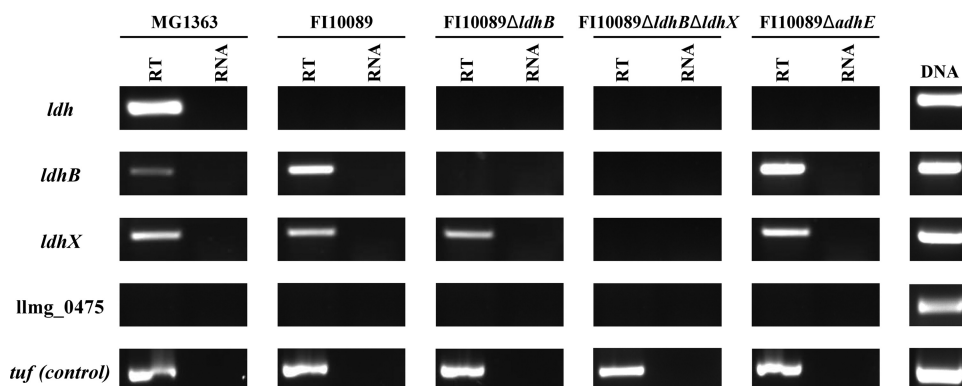


FIG. 2. Transcriptional studies of lactate dehydrogenase genes in *L. lactis* MG1363 and derivatives grown in CDM with 1% glucose (wt/vol) and without pH control (initial pH 6.5). RT-PCR experiments were performed using primers to amplify intragenic regions of the *ldh* (389 bp), *ldhB* (417 bp), *ldhX* (449 bp), *llmg_0475* (474 bp), and control *tufA* (471 bp) genes. “RT” and “RNA” indicate PCRs performed on total RNA with or without reverse transcriptase treatment, respectively, the latter acting as a negative control. “DNA” indicates positive PCR controls performed with MG1363 chromosomal DNA as the template.

cysteine-HCl buffers. The levels of MTLD, MTLF, ALS, and AR overproduction were determined with cell extracts prepared from cultures in the late-exponential growth phase. Cells were grown as described above, harvested by centrifugation ($2,136 \times g$, 5 min, 4°C), washed twice with KPi 50 mM (pH 6.0) or 2-[*N*-morpholino]ethane-sulfonic acid/KOH (MES/KOH) buffer, pH 6.0, and resuspended in the same buffers to a final protein concentration ranging from 4 to $10 \text{ mg} \cdot \text{ml}^{-1}$. Crude extracts were prepared by passage through a French press (twice at 120 MPa) and centrifugation for 15 min at $30,000 \times g$ and at 4°C to remove cell debris. Alternatively, disruption using glass beads ($\leq 106 \mu\text{m}$) and a MiniBeadbeater-8 cell disrupter (Biospec Products), 2 cycles of 1 min each at the homogenize position, was performed. After disruption, the extracts were centrifuged at $16,100 \times g$ for 20 min at 4°C . All enzyme activities were assayed at 30°C in a Beckman Coulter DU 800 spectrophotometer. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of $1 \mu\text{mol}$ of substrate per minute under the experimental conditions used. The protein concentration was determined by the method of Bradford (7). Specific activity was expressed as units ($\mu\text{mol} \cdot \text{min}^{-1}$) per milligram of protein ($\text{U} \cdot \text{mg}$ of protein $^{-1}$).

The *las* gene-encoded LDH activity was assayed as described by Garrigues et al. (17). Lactate dehydrogenase activity encoded by the *ldhB* gene was monitored as LDH but at pH 6.0 in 100 mM MES/KOH buffer (20). Alcohol dehydrogenase (ADHE) was determined as described by Even et al. (14). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity was determined as described by Even et al. (13), after 1 h of incubation at 30°C in 125 mM triethanolamine buffer (pH 7.2) with 5 mM cysteine-HCl to reactivate the enzyme by reducing the sulfhydryl groups. The reaction mixture contained 125 mM triethanolamine buffer (pH 7.2), 5 mM cysteine-HCl, 40 mM arsenate, 6 mM glyceraldehyde 3-phosphate and 5 mM NAD^{+} . The backward reaction catalyzed by MTLD was assayed as reported before (41) in a reaction mixture containing 100 mM Tris-HCl buffer (pH 7.2), 0.3 mM NADH, and 3 mM fructose 6-phosphate. For determination of MTLF, cells were suspended in 50 mM/KOH buffer, pH 7.0. Phosphatase activity was assayed as described by Neves et al. (41) in a reaction mixture consisting of 50 mM MES/KOH (pH 7.0), 10 mM MgCl_2 , and 3 mM mannitol 1-phosphate. The reaction was stopped several times after the addition of mannitol 1-phosphate by mixing 0.7 ml of phosphate reagent (1 part 10% ascorbic acid solution and 6 parts 0.42% ammonium molybdate in 1 N H_2SO_4) with 0.3 ml of reaction mixture, and the phosphate was measured colorimetrically at 820 nm by the method described by Ames (1). ALS activity was determined as described by Hugenoltz and Starrenburg (26). The reaction was stopped by pH decrease (addition of 100 μl of 6 N H_2SO_4), and acetoin was quantified colorimetrically at 525 nm as described by Westerfeld (59). AR was assayed as described by Stormer (54), in a modified reaction mixture containing 50 mM KPi (pH 6.0), 0.2 mM NADH, and 2.5 mM acetoin.

NMR spectroscopy. The isomeric forms of 2,3-butanediol were determined by ^{13}C -nuclear magnetic resonance (^{13}C -NMR) analysis using a Bruker DRX500 spectrometer and a 5-mm selective probe head.

RESULTS

Expression of lactate dehydrogenase-encoding genes in *L. lactis* MG1363 and *ldh*-deficient (FI10089) strains. In addition to the *ldh* (*las* operon) and *ldhB* (20, 37) genes, two other genes potentially encoding L-lactate dehydrogenases, i.e., the *ldhX* and *llmg_0475* genes, are present in the genome of *L. lactis* MG1363 (58). Genes with homology to D-lactate dehydrogenases were not found. In this study, we used RT-PCR to examine the expression of those four genes in MG1363 and its derivative strain FI10089 (19). The *ldh*, *ldhB*, and *ldhX* genes were transcribed in MG1363 (Fig. 2). The *ldhX* gene was expressed to a similar extent in the *ldh*-deficient strain and MG1363, but *ldhB* gene expression was clearly higher in the former strain. In contrast, the *llmg_0475* gene was not expressed in either strain.

Inactivation of the *ldhB*, *ldhX*, and *adhE* genes in *L. lactis* FI10089 by double-crossover recombination. Induction of alternative *ldh* genes may hinder redirection of the carbon flux in *L. lactis* toward products other than lactate. Therefore, *ldhB* and *ldhX* genes were sequentially deleted by double-crossover recombination from the genome of FI10089, generating strains FI10089 Δ *ldhB* and FI10089 Δ *ldhB* Δ *ldhX*, respectively (Fig. 1). Earlier work by our team showed that ethanol was the major end product of glucose metabolism by the *ldh*-deficient strain, FI10089, accounting for approximately 40% of the glucose consumed (19). Therefore, we investigated the effect of inactivating the gene encoding alcohol dehydrogenase on the profile of end products. To this end, the *adhE* gene was deleted in FI10089 to generate strain FI10089 Δ *adhE*.

The impact of the deletions in strain FI10089 on the expression of genes encoding characterized (*ldh* and *ldhB*) or potential (*ldhX* and *llmg_0475*) lactate dehydrogenases was also investigated by RT-PCR (Fig. 2). Deletion of the *ldhB* or *adhE* genes in FI10089 did not affect the expression level of the *ldhX* gene. However, the expression of the *ldhB* gene increased markedly in FI10089 Δ *adhE*. Expression of the *llmg_0475* gene was not detected in any of the deletion strains constructed in this study.

TABLE 1. Specific activities of NADH-dependent dehydrogenases in crude cell extracts of MG1363 and mutant strains with deletions of genes encoding lactate dehydrogenase and alcohol dehydrogenase

Strain	Mean sp act of enzyme \pm SD (U \cdot mg of protein ⁻¹) ^a					
	LDH		ADHE	MTLD	AR	GAPDH
	pH 7.2	pH 6.0				
MG1363	22.11 \pm 0.64	26.15 \pm 1.32	0.01 \pm 0.00	ND	0.02 \pm 0.00	29.70 \pm 0.80 ^b
FI10089	ND	0.23 \pm 0.02	0.51 \pm 0.04	2.98 \pm 0.24	0.04 \pm 0.01	30.29 \pm 1.84
FI10089 Δ ldhB	ND	ND	0.48 \pm 0.11	0.04 \pm 0.00	0.04 \pm 0.01	33.84 \pm 0.28
FI10089 Δ ldhB Δ ldhX	ND	ND	0.57 \pm 0.05	0.01 \pm 0.00	0.05 \pm 0.01	28.73 \pm 2.40
FI10089 Δ adhE	0.31 \pm 0.04	22.44 \pm 5.22	ND	1.68 \pm 0.19	0.02 \pm 0.00	27.41 \pm 3.39

^a Activity was determined at least twice for two extracts derived from independent cultures. LDH, lactate dehydrogenase; ADHE, alcohol dehydrogenase; MTLD, mannitol 1-phosphate dehydrogenase; AR, acetoin reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ND, below the detection limit.

^b Data from Neves et al. (43).

Effect of specific gene inactivation on the levels of NAD⁺-dependent dehydrogenases involved in glucose fermentation. NAD⁺-dependent dehydrogenase activities were measured in crude extracts derived from cultures of MG1363, FI10089, FI10089 Δ ldhB, FI10089 Δ ldhB Δ ldhX, and FI10089 Δ adhE (Table 1). The specific activity of lactate dehydrogenase was measured at pH 7.2 and 6.0 in order to distinguish between the activities encoded by the *ldh* and *ldhB* genes. While the *ldh*-encoded enzyme shows a maximal activity between pH 5.2 and 7.2, LDHB displays highest activity in a pH range of 5.5 to 6.0 (20). MG1363 displayed high lactate dehydrogenase activity regardless of pH. In contrast, this activity was reduced about 100-fold in the *ldh*-deficient strain FI10089 and was detectable only at pH 6.0, which defines the activity as LDHB. Strains FI10089 Δ ldhB and FI10089 Δ ldhB Δ ldhX did not exhibit lactate dehydrogenase activity at any of the pH values examined. In FI10089 Δ adhE, the activity at pH 6.0 was 72-fold higher than that measured at pH 7.2, which correlates with the increase in the expression of the *ldhB* gene compared to that in the parental strain FI10089 (Fig. 2).

The ADHE activity was similar in strains FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX (0.5 U \cdot mg of protein⁻¹) and about 50-fold lower in MG1363. The MTLD activity was detected only in LDH-deficient strains. However, it decreased from 3 U \cdot mg of protein⁻¹ in FI10089 to 0.04, 0.01, and 1.7 U \cdot mg of protein⁻¹ in FI10089 Δ ldhB, FI10089 Δ ldhB Δ ldhX, and FI10089 Δ adhE, respectively. All the strains examined showed similar AR (0.02 to 0.05 U \cdot mg of protein⁻¹) and GAPDH (\sim 30 U \cdot mg of protein⁻¹) activities.

Characterization of anaerobic glucose metabolism by strains FI10089 Δ ldhB, FI10089 Δ ldhB Δ ldhX, and FI10089 Δ adhE. The growth profiles of strains FI10089 Δ ldhB, FI10089 Δ ldhB Δ ldhX, and FI10089 Δ adhE in CDM supplemented with 1% glucose at pH 6.5, under anaerobic conditions, were studied (Fig. 3). The double and triple lactate dehydrogenase-deficient strains showed growth profiles similar to FI10089, whereas FI10089 Δ adhE displayed a higher growth rate. A comparison of growth rate, product yield, and carbon balance for the different strains is presented in Table 2. Strains FI10089 Δ ldhB and FI10089 Δ ldhB Δ ldhX retained a mixed-acid fermentation profile as FI10089 (19). These three strains produced formate, ethanol, acetate, acetoin, and 2,3-butanediol as well as minor amounts of lactate and mannitol. In addition, the double and triple lactate dehydrogenase-deficient strains accumulated pyruvate.

Lactate production was progressively decreased with the cumulative deletion of lactate dehydrogenase-encoding genes. The *ldh*-negative strain, FI10089, produced about 20-fold less lactate than the parent strain MG1363, corresponding to 0.09 mol of lactate per mole of glucose consumed. The sequential deletion of *ldhB* and *ldhX* genes in FI10089 led to a further reduction in lactate, but the production of this acid was not fully abolished in the triple-deletion mutant.

Deletion of the *adhE* gene in FI10089 caused a marked metabolic shift from mixed-acid to homolactic fermentation, resembling the metabolic profile of the parental strain MG1363. Lactate was by far the main end product from glucose metabolism and only small amounts of acetate, formate, mannitol, and acetoin were formed. In view of these results, the *ldhB* gene was deleted in FI10089 Δ adhE, yielding strain FI10089 Δ adhE Δ ldhB (see Table S1 in the supplemental material); as expected, this strain was not able to grow under anaerobic conditions. The strain was used as a host for the expression of genes involved in mannitol biosynthesis (see below), but the NAD⁺-regeneration capacity provided by this manipulation remained insufficient to permit growth under anaerobic conditions (data not shown).

Lactate dehydrogenase-deficient strains engineered for the production of mannitol and 2,3-butanediol. In view of the

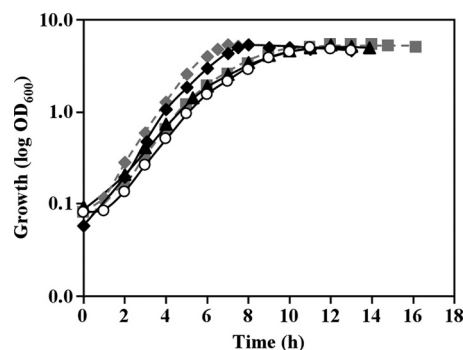


FIG. 3. Growth of lactate dehydrogenase-deficient strains in CDM supplemented with 1% glucose (wt/vol) at 30°C and pH 6.5. Symbols: black triangles, FI10089 Δ ldhB; white circles, FI10089 Δ ldhB Δ ldhX; black diamonds, FI10089 Δ adhE; gray squares, FI10089 Δ ldhB Δ ldhX Δ adhE; gray diamonds, MG1363 (data from Neves et al. [42]). Data are from a representative experiment. Growths were repeated at least twice for each strain. Lines are simple interpolations.

TABLE 2. Growth rate, product yield, and carbon balance for glucose metabolism by *L. lactis* MG1363 and derivative strains^h

Strain	μ^a (h ⁻¹)	Product yield \pm SD (mol \cdot mol of glucose ⁻¹)								C_{bal}^c (%)
		Lactate	Formate	Ethanol	Acetate	2,3-Bd ^b	Acetoin	Mannitol	Pyruvate	
MG1363 ^d	0.78	1.80	0.07	0.06	0.02	ND ^e	ND	ND	ND	95
FI10089 ^e	0.66 \pm 0.01	0.09 \pm 0.00	1.33 \pm 0.18	0.81 \pm 0.12	0.44 \pm 0.06	0.10 \pm 0.01	0.16 \pm 0.02	0.04 \pm 0.01	ND	98 \pm 3
FI10089 Δ ldhB ^f	0.58 \pm 0.01	0.05 \pm 0.00	1.34 \pm 0.01	0.79 \pm 0.01	0.46 \pm 0.01	0.15 \pm 0.00	0.10 \pm 0.00	0.005 \pm 0.000	0.02 \pm 0.00	90 \pm 0
FI10089 Δ ldhB Δ ldhX ^f	0.61 \pm 0.01	0.03 \pm 0.00	1.23 \pm 0.03	0.83 \pm 0.02	0.35 \pm 0.02	0.09 \pm 0.01	0.18 \pm 0.00	0.003 \pm 0.001	0.04 \pm 0.00	91 \pm 1
FI10089 Δ adhE ^f	0.75 \pm 0.01	1.77 \pm 0.01	0.02 \pm 0.00	ND	0.03 \pm 0.00	ND	0.01 \pm 0.00	0.01 \pm 0.00	ND	92 \pm 0

^a μ , instant growth rate.
^b 2,3-Bd, 2,3-butanediol.
^c Carbon balance (C_{bal}) is the percentage of carbon in metabolized glucose that is recovered in the fermentation products and calculated as follows: $\{[mannitol] + ([2,3-butanediol] \times 2 + [acetoin] \times 2 + [lactate] + [acetate] + [ethanol] + [pyruvate])/2\} / [glucose]_c \times 100$, where $[glucose]_c$ corresponds to the glucose consumed during the period of monitoring.
^d Data from Neves et al. (42).
^e Data from Gaspar et al. (19).
^f Average values from at least two independent growths. Data from individual experiments did not differ by more than 16% (product yield) or 2% (growth rate and carbon balance).
^g ND, below the detection limit.
^h The pH of the cultures was maintained at 6.5.

metabolic profiles described above, FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX were selected as hosts for the overproduction of the mannitol and 2,3-butanediol biosynthetic pathways. The lactococcal *mtlD* gene, encoding MTLT, and the heterologous gene coding for a specific MTLT from *Eimeria tenella* were cloned under the control of the nisin-inducible promoter P_{nisA} to achieve high expression of the mannitol pathway. The latter gene was used because the gene encoding MTLT in *L. lactis* has not been identified. A similar strategy was followed to overexpress the lactococcal genes encoding ALS (*als*) and AR (*butA*), thereby maximizing the production of 2,3-butanediol. To evaluate if the enzymes were functionally produced, specific activities were measured with the relevant cell extracts (see Table S4 in the supplemental material). The MTLT and MTLT activities were similar among all mannitol-overproducing strains, with average values of 8 and 2 U \cdot mg of protein⁻¹, respectively, which are considerably higher than those for control strains. Induced expression of the *als* and *butA* genes in the selected hosts led to a >130-fold increase in ALS and AR activities compared to results for control strains.

The effect of overproducing mannitol and 2,3-butanediol biosynthetic pathways on the growth properties of constructed strains was evaluated using glucose as the carbon source (Fig. 4 and Table 3). The growth rate and maximal biomass of the host strains were negatively affected by overexpression of the mannitol pathway. Despite this effect, 30 to 42% of the glucose consumed was converted to mannitol (Table 4) (see Fig. S1 in the supplemental material). This corresponds to an increase of 10-, 42-, and 30-fold in the yield of mannitol relative to levels in the host strains FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX, respectively. In the mannitol overproducers, the levels of 2,3-butanediol, acetoin, lactate, formate, and ethanol decreased, while those of acetate increased or did not change. A higher percentage of glucose was consumed by overproducer strains (Table 4) than by controls. As expected, redirection of the carbon flux to mannitol production led to a decrease in the ATP yield (Table 3).

Strains overexpressing the 2,3-butanediol biosynthetic pathway displayed higher growth rates and maximal biomass than

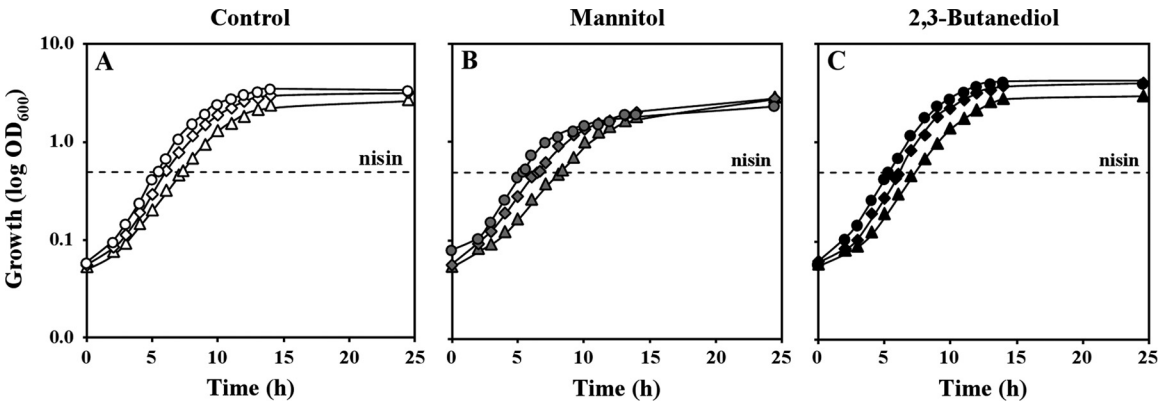


FIG. 4. Growth of FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX overproducing the mannitol (B) or the 2,3-butanediol (C) biosynthetic pathway compared to that of control strains containing the expression vector pNZ8048 and the auxiliary plasmid pNZ9530 (A). Strains were grown in CDM supplemented with 1% glucose (wt/vol) at 30°C without pH control (initial pH 6.5) under static conditions. Erythromycin (5 mg \cdot liter⁻¹) and chloramphenicol (5 mg \cdot liter⁻¹) were added to the growth medium to maintain plasmid selection. To induce expression of mannitol and 2,3-butanediol biosynthetic genes, nisin (1 μ g \cdot liter⁻¹) was added at OD₆₀₀ of 0.5. Symbols: diamonds, FI10089; circles, FI10089 Δ ldhB; triangles, FI10089 Δ ldhB Δ ldhX. Average curves from at least two independent growths for each strain. The values of OD₆₀₀ in each time point did not differ by more than 16%. Lines are simple interpolations.

TABLE 3. Growth rate, energy parameters, and carbon balance obtained for strains FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX overexpressing the mannitol or the 2,3-butanediol biosynthetic pathway^a

Parameter	Control ^d			Mannitol pathway			2,3-Butanediol pathway		
	FI10089	FI10089 Δ ldhB	FI10089 Δ ldhB Δ ldhX	FI10089	FI10089 Δ ldhB	FI10089 Δ ldhB Δ ldhX	FI10089	FI10089 Δ ldhB	FI10089 Δ ldhB Δ ldhX
μ_1^b (h ⁻¹)	0.46 ± 0.01	0.49 ± 0.01	0.37 ± 0.01	0.38 ± 0.00	0.47 ± 0.02	0.33 ± 0.02	0.45 ± 0.01	0.49 ± 0.01	0.42 ± 0.01
μ_2^b (h ⁻¹)	0.37 ± 0.01	0.43 ± 0.02	0.35 ± 0.02	0.31 ± 0.00	0.22 ± 0.01	0.31 ± 0.01	0.44 ± 0.04	0.48 ± 0.02	0.37 ± 0.02
ATP yield (mol · mol of glucose ⁻¹)	2.0 ± 0.02	2.1 ± 0.04	2.0 ± 0.03	1.3 ± 0.01	1.2 ± 0.13	1.5 ± 0.01	2.0 ± 0.05	2.1 ± 0.04	2.1 ± 0.02
Y _{ATP} (g of biomass · mol of ATP ⁻¹)	18.8 ± 0.3	17.7 ± 0.4	15.2 ± 0.5	16.0 ± 0.1	13.1 ± 0.2	15.5 ± 0.1	18.1 ± 0.1	16.5 ± 0.2	14.7 ± 0.0
Carbon balance ^c (%)	95 ± 1	94 ± 2	94 ± 1	94 ± 0	96 ± 1	94 ± 1	102 ± 2	104 ± 2	102 ± 1

^a Cells were grown without pH control (initial pH 6.5). Shown are average values ± standard deviations from at least two independent growths. Data from individual experiments did not differ by more than 13% (growth rate), 11% (ATP yield), 3% (Y_{ATP}), or 2% (carbon balance).

^b μ_1 and μ_2 , growth rates before and after nisin addition (1 μ g · liter⁻¹), respectively.

^c Carbon balance is the percentage of carbon in metabolized glucose that is recovered in fermentation products and calculated as follows: $\{[\text{mannitol}] + ([2,3\text{-butanediol}] \times 2 + [\text{acetoin}] \times 2 + [\text{lactate}] + [\text{acetate}] + [\text{ethanol}] + [\text{pyruvate}]) / 2\} / [\text{glucose}] \times 100$, where [glucose] corresponds to the glucose consumed during the period of monitoring.

^d Strains harboring empty plasmids.

those of their respective controls (Fig. 4 and Table 3). Remarkably, 65 to 67% of the substrate consumed was converted to 2,3-butanediol, which corresponds to an increase of 5- to 8-fold in the yield of 2,3-butanediol compared to the host strains FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX. ¹³C-NMR analysis of culture supernatants revealed that *meso*-2,3-butanediol was the only isomeric form present (data not shown). The levels of the other end products (lactate, acetoin, formate, ethanol, acetate, and pyruvate) were significantly reduced in strains overexpressing the 2,3-butanediol biosynthetic genes (Table 4) (see Fig. S1 in the supplemental material). Moreover, the percentage of glucose consumed was greater and the ATP yields were similar (Tables 3 and 4).

DISCUSSION

Lactococcal metabolism is tuned for maximal production of lactic acid from glucose, permitting rapid NAD⁺ recycling at the level of lactate dehydrogenase. For this reason, metabolic engineering of *L. lactis* for the biotechnological production of reduced compounds requires strains with strong impairment in the lactate dehydrogenase activity (19, 25, 45, 60). Inactivation

of the *las*-located *ldh* gene, encoding the main lactate dehydrogenase activity, led to considerable reduction of lactate production, but even so, this organic acid was invariably present among the fermentation products. Curiously, in some cases, inactivation of the *ldh* gene resulted in strains displaying a homolactic metabolism, similar to that of the wild-type strains. This surprising result is due to activation of the *ldhB* gene, which codes for an alternative lactate dehydrogenase (5, 20). In view of this unforeseen activation, we decided to study the expression of known (*ldh* and *ldhB*) and putative (*ldhX* and *lmg_0475*) lactate dehydrogenase genes in the parent strain MG1363 and in a derivative with a deletion in the *ldh* gene (strain FI10089), which was used here as background for further genetic manipulation. Transcripts of the *ldhB* and *ldhX* genes were detected in both strains. In contrast, *ldhB* and *ldhX* transcripts were not detected in *L. lactis* NZ9000 and its *ldh*-deficient derivative (5), despite the common origin of these strains and FI10089, which are all derived from the prototype strain MG1363 that is widely used for LAB genetics. In another study, the expression patterns of the *ldhB* and *ldhX* genes were not differentially affected in MG1363 and NZ9000 (36);

TABLE 4. Product yield determined for strains FI10089, FI10089 Δ ldhB, and FI10089 Δ ldhB Δ ldhX overexpressing the mannitol or the 2,3-butanediol biosynthetic pathway^a

Product yield (mol · mol of glucose ⁻¹)	Control ^b			Mannitol pathway			2,3-Butanediol pathway		
	FI10089	FI10089 Δ ldhB	FI10089 Δ ldhB Δ ldhX	FI10089	FI10089 Δ ldhB	FI10089 Δ ldhB Δ ldhX	FI10089	FI10089 Δ ldhB	FI10089 Δ ldhB Δ ldhX
Mannitol	0.04 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.38 ± 0.00	0.42 ± 0.00	0.30 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
2,3-Butanediol	0.10 ± 0.01	0.15 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.65 ± 0.01	0.67 ± 0.01	0.67 ± 0.01
Acetoin	0.26 ± 0.01	0.21 ± 0.01	0.31 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.25 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Lactate	0.06 ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Formate	1.00 ± 0.02	1.06 ± 0.04	0.99 ± 0.03	0.51 ± 0.00	0.50 ± 0.01	0.61 ± 0.01	0.65 ± 0.03	0.74 ± 0.08	0.65 ± 0.00
Acetate	0.21 ± 0.00	0.22 ± 0.01	0.14 ± 0.01	0.22 ± 0.00	0.22 ± 0.01	0.17 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
Ethanol	0.78 ± 0.01	0.80 ± 0.02	0.83 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.44 ± 0.00	0.56 ± 0.02	0.61 ± 0.01	0.59 ± 0.01
Pyruvate	0.06 ± 0.00	0.09 ± 0.01	0.08 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.06 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Glucose consumed (%)	48 ± 1	55 ± 3	47 ± 1	56 ± 1	66 ± 1	49 ± 0	62 ± 2	71 ± 3	56 ± 2

^a Cells were grown without pH control (initial pH 6.5). Shown are average values ± standard deviations from at least two independent growths. Data from individual experiments did not differ by more than 16% (product yield) or 5% (glucose consumed) and were calculated at 14 h.

^b Strains harboring empty plasmids.

hence, we speculate that the dissimilarity arises from different growth conditions and/or methodologies to assess expression. Recently, Cao et al. (8) cited unpublished results with *L. lactis* strain ATCC19435 showing concomitant expression of the *ldh*, *ldhB*, and *ldhX* genes.

Taking into consideration the observed *ldhB* and *ldhX* expression profiles (Fig. 2), these genes were deleted in FI10089. Furthermore, the remarkable induction of the *ldhB* gene in response to *adhE* gene inactivation in FI10089 strongly supports the need for an *ldhB* null mutant as a host for metabolic engineering strategies intended to limit the capacity for NAD⁺ regeneration. Indeed, loss of the *adhE* gene, encoding alcohol dehydrogenase, reverted the fermentation profile of FI10089 to fully homolactic (Table 2). Curiously, the molecular mechanisms leading to induction of the *ldhB* gene in *L. lactis*, namely, insertion of IS and/or point mutations in the promoter region (5, 20), do not seem to have taken place here, since a sequence analysis did not show any difference in the *rlrD*-*ldhB* intergenic region of FI10089 Δ *adhE* and MG1363 (data not shown). Therefore, the molecular basis for induction of the *ldhB* gene, triggered by disruption of alcohol dehydrogenase, remains elusive.

In contrast to the case with the *ldhB* gene, expression of the *ldhX* gene is apparently constitutive in *L. lactis*. Additionally, the contribution of its gene product to the reduction of pyruvate to lactate is negligible, as shown by the small decrease in the lactate yield (from 0.05 to 0.03 mol · mol of glucose⁻¹), when the *ldhX* gene was deleted in FI10089 Δ *ldhB*. It should be noted that, inactivation of the *ldhB* gene in FI10089 caused also a small reduction in the lactate yield (from 0.09 to 0.05 mol · mol of glucose⁻¹), while disruption of the *ldh* gene decreased the lactate yield 20-fold (from 1.8 to 0.09 mol · mol of glucose⁻¹). Therefore, the *ldh* gene encodes by far the major lactate dehydrogenase activity in *L. lactis* MG1363, and the contributions of the alternative lactate dehydrogenases are small.

Curiously, cumulative inactivation of *ldh*, *ldhB*, and *ldhX* genes did not completely abolish lactate production (Table 2). However, lactate synthesis by Lmg_0475 can be excluded, since expression of its encoding gene was not detected. Production of lactate by other members of LAB accumulating serial deletions of lactate dehydrogenase genes have also been reported, but the identity of the underlying activities was not investigated (15, 29, 48). We failed to detect lactate dehydrogenase activity in the triple mutant (FI10089 Δ *ldhB* Δ *ldhX*), despite examinations at different pH values and replacement of NADH by NADPH (data not shown). Therefore, identification of the protein by purification of the activity is impossible. BLASTp (<http://www.ncbi.nlm.nih.gov/genomes/gebblast.cgi?gi=20494>) searches of the MG1363 genome using as queries sequences of functional D-lactic acid dehydrogenases, NADH-independent lactate dehydrogenases or specific motifs of the lactate dehydrogenase clade of a new family of NAD(P)H-dependent oxidoreductases, revealed no homologs (18, 40). Therefore, the identity of the protein(s) responsible for residual lactate production in FI10089 Δ *ldhB* Δ *ldhX* remains unknown. Considering the low level of lactate detected (2 mM lactate from 55 mM glucose), the involvement of nonspecific dehydrogenase(s) seems plausible (2, 48). Additionally, lactate production could be due to the action of the malolactic enzyme

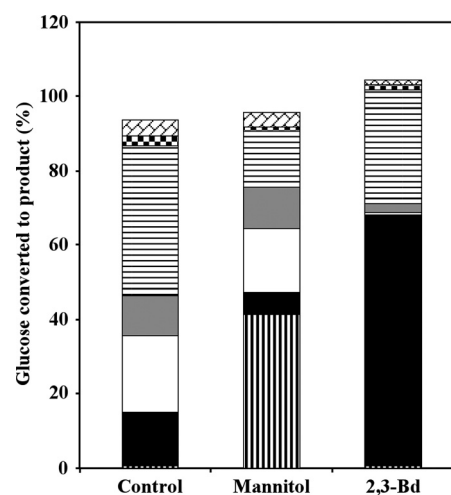


FIG. 5. Percentage of glucose converted to end product by FI10089 Δ *ldhB* overproducing the mannitol or the 2,3-butanediol (2,3-Bd) biosynthetic pathways, compared to the control strain containing the expression vector pNZ8048 and the auxiliary plasmid pNZ9530. Strains were grown in CDM supplemented with 1% glucose (wt/vol) at 30°C without pH control (initial pH 6.5) under static conditions. Erythromycin (5 mg · liter⁻¹) and chloramphenicol (5 mg · liter⁻¹) were added to the growth medium to maintain plasmid selection. To induce expression of mannitol and 2,3-butanediol biosynthetic genes, nisin (1 µg · liter⁻¹) was added at OD₆₀₀ of 0.5. Patterns: vertical stripes, mannitol; solid black, 2,3-butanediol; white, acetoin; gray, acetate; horizontal stripes, ethanol; checkered, lactate; diagonal bricks, pyruvate. Formate was also produced (Table 4) but is not displayed.

(*mleS*, lmg_1638) which is able to convert oxaloacetate into lactate via malate (47).

The criteria used to select the best production host were their genetic stability and their high reducing potential. While strains FI10089 Δ *ldhB* and FI10089 Δ *ldhB* Δ *ldhX* met both criteria, strain FI10089 satisfied only the second one. Nevertheless, we assessed the feasibility of these three strains as hosts for engineering the production of mannitol and 2,3-butanediol. These polyols are by-products of glucose metabolism in *ldh*-deficient strains (19, 41), but we showed that the respective yields can be remarkably improved through overproduction of the relevant biosynthetic enzymes. Maximal mannitol and 2,3-butanediol yields were achieved when strain FI10089 Δ *ldhB* was used as a host (Fig. 5). In fact, about 42% of the glucose was channeled to mannitol, a value close to the theoretical maximum of 50%, calculated on the assumption that glucose is transported via phosphoenolpyruvate phosphotransferase systems only (9). Higher mannitol yields have been reported in *L. lactis* NZ9010 by Wisselink et al. (61), using a slightly different engineering strategy. Most likely, the reason for the enhanced mannitol production lies in the distinct fermentation conditions used in the two studies (rich medium versus CDM, higher glucose content, different host strains, etc.). It is worth noting that our main goal was not the optimization of mannitol production, but rather the investigation of the impact on cell physiology caused by manipulation of the NAD⁺ recycling capacity.

To our knowledge, this is the first work reporting *L. lactis* strains engineered for the production of 2,3-butanediol. Interestingly, the overproducer strains reached the maximum the-

oretical 2,3-butanediol yield predicted for glucose metabolism under anaerobic conditions (67%) without a special effort to manipulate fermentation conditions. In this context, *L. lactis* is a promising candidate for the production of 2,3-butanediol from inexpensive substrates, such as whey permeate, a by-product of cheese manufacture. A great research effort has been directed to improve the 2,3-butanediol productivity and yield in native producers, such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, or *Paenibacillus polymyxa* (for recent reviews, see references 10 and 27). However, the pathogenic status of these species is an obstacle to their utilization in industrial-scale fermentation processes due to health concerns. Alternatively, production of 2,3-butanediol has been engineered in *Escherichia coli*, a nonnative producer that requires the reconstruction of the complete biosynthetic pathway (34, 44, 56, 57, 62). The present work establishes *L. lactis* as an additional producer of potential importance for the transformation of waste products derived from the dairy industry.

Overexpression of the mannitol and 2,3-butanediol biosynthetic pathways induced notable and opposite changes in the growth profiles of hosts strains: 2,3-butanediol producers had higher growth rates and maximal biomass, whereas mannitol overproduction led to a substantial decrease in both parameters (Fig. 4 and Table 3). This result was not unexpected, since the node for redirection of the carbon flux occurs upstream (mannitol) or downstream (2,3-butanediol) of ATP-generating steps, respectively. In the case of mannitol production, however, a metabolic burden due to high expression levels of a foreign protein (MTLP) cannot be excluded (6). Nevertheless, limitation of anabolic processes and, consequently, growth by an ATP shortage is further supported by the low ATP yields in mannitol producers (Table 3). In agreement, decreased ethanol/acetate and 2,3-butanediol/acetoin ratios indicate that the pressure to generate ATP surpassed that of recycling NAD⁺. Thus, mannitol-producing strains may benefit from further manipulations to increase ATP supply.

LDH-deficient strains are characterized by a bottleneck at the level of NAD⁺ regeneration (43). Overexpression of the 2,3-butanediol biosynthetic pathway seems to overcome this limitation on NADH oxidation as shown by the higher growth rates of overproducer strains (Table 3). Respectively, the high activities of ALS and AR in engineered strains enhance pyruvate utilization and lead to an efficient oxidation of the NADH generated during glycolysis. 2,3-Butanediol overproducer strains also grew to higher ODs than control strains, most likely due to lower acidification of the culture medium of strains producing high levels of the polyol. Indeed, the medium pH decreased by about 0.6 units in 2,3-butanediol producers, while control strains registered a 1 unit decrease.

In *L. lactis*, the *butA* gene (annotated as an acetoin reductase gene) forms an operon with the *butB* (annotated as a 2,3-butanediol dehydrogenase gene), whose transcription is driven from the weak promoter P_{butBA} (16). Expression of the *butA* gene in the different host strains resulted in the production of the optically inactive form of 2,3-butanediol, i.e., *meso*-2,3-butanediol, as determined by ¹³C-NMR analysis of fermentation products. In a recent work, the *butB* gene was expressed in *E. coli*, and the engineered strain produced *meso*-2,3-butanediol (44). The definite characterization of the reactions catalyzed by ButA and ButB will demand assays with pure gene

products, as the apparently identical roles assigned to ButA and ButB might be masked by the presence of racemase activities *in vivo*. However, the high amino acid sequence identity of the lactococcal enzyme encoded by the *butA* gene with an acetoin reductase (BudC) of *K. pneumoniae* (65% identity), which synthesizes *meso*-2,3-butanediol from D-acetoin (55), leads to the suggestion that the *butA* gene codes for an enzyme with identical activity in *L. lactis*.

In conclusion, the engineering strategy pursued here led to the development of *L. lactis* strains with high potential for the production of reduced compounds, such as polyols. The combined deletion of *ldh* and *ldhB* genes was essential to achieve a stable metabolic performance. Moreover, the deletion of the remaining lactate dehydrogenase-encoding gene, the *ldhX* gene, was superfluous, since polyol production was not improved. *L. lactis* is proposed as a platform for the conversion of waste products from the dairy industry into 2,3-butanediol, a valuable chemical feedstock.

ACKNOWLEDGMENTS

This work was supported by the Fundação para a Ciência e a Tecnologia (FCT), contracts PTDC/EEA-ACR/69530/2006 and PTDC/EBB-EBI/113727/2009. Paula Gaspar acknowledges FCT for the award of a postdoctoral grant (SFRH/BPD/31251/2006).

We thank Paul Liberatore (Merck Research Laboratories) and Michiel Kleerebezem (NIZO food research) for supplying the pQE60 plasmid containing the *mlp* gene from *Eimeria tenella* and the pNZ9530 plasmid, respectively.

The NMR spectrometers are part of the National NMR Network (REDE/1517/RMN/2005), supported by "Programa Operacional Ciência e Inovação (POCTI) 2010" and FCT.

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